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Certified by

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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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Additional inventors are being named on the _____ separately numbered sheets attached hereto

TITLE OF THE INVENTION (500 characters max)

METHODS AND COMPOSITIONS FOR INDUCING FOLLICLE MATURATION

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- [ ] Applicant claims small entity status. See 37 CFR 1.27.
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[X] No [ ] Yes, the name of the U.S. Government agency and the Government contract number are: _____

Respectfully submitted,

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Dated: January 28, 2004 Signature: [signature] (Ernesto Del Real)

Docket No.: 30694/39707

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
PROVISIONAL APPLICATION FOR UNITED STATES LETTERS PATENT

PROVISIONAL APPLICATION COVER SHEET FOR APPLICATION OF TITLE:

METHODS AND COMPOSITIONS FOR INDUCING FOLLICLE MATURATION

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Dated: January 28, 2004 Signature: _____ (Ernesto Del Real)

METHODS AND COMPOSITIONS FOR INDUCING FOLLICLE MATURATION

Field of the Invention

The present invention is generally directed to reproductive biology. More specifically, the present invention is directed to methods and compositions for inducing follicle maturation using one or more phosphodiesterase inhibitors either alone or in combination with one or more gonadotropins.

Background of the Related Art

The female human reproductive cycle relies on a number of gonadotropins hormones. Principle amongst these are the pituitary hormones follicle stimulating hormone, (FSH) and luteinizing hormone (LH). During oogenesis, the process by which the female germ cell, the ovum, is produced occurs within a follicle. A follicle is a collection of cells in the ovary containing an oocyte (egg). Follicle maturation which ultimately leads to ovulation, is dependent on the stimulatory effects of FSH.

In each menstrual cycle, many follicles are recruited for the maturation of the oocytes. At the beginning of the approximately 28-day menstrual cycle the follicles are in the primordial form, which is simply an oocyte surrounded by a single layer of cells. As follicular growth and maturation is activated by FSH, multiple layers of granulosa cells form around the initial single layer of cells, a process that continues through to midcycle. These granulosa cells are responsible for nourishing the oocyte and for the production and release of estrogen. FSH, produced by the pituitary induces aromatase activity in the granulosa cells thereby increasing the production of estrogen. Thus, concurrent with the maturation of a follicle there is an increase in estrogen production in the early part of the 28-day menstrual cycle. The follicle also contains receptors for the second pituitary gonadotropin, LH. As the follicle continues to grow and mature by mid-cycle (approx. day 14), a space (antrum) develops inside the granulosa cells. At mid-cycle a surge of LH production acts on LH receptors to cause the follicle to rupture and release the oocyte which travels down the fallopian tube and, which may subsequently be fertilized.

The normal ovulating woman recruits approx. 300 immature oocytes for each menstrual cycle. During a normal cycle, all but one follicle will regress

(atresia), and a single dominant follicle will emerge and go on to release an oocyte. *In vitro* fertilization (IVF) of human oocytes, which is now a commonly used treatment for female and male subfertility, is based on retrieval of mature human oocytes followed by fertilization of the mature oocytes with spermatozoa. The recruitment of human mature oocytes is accomplished by hormone treatment regimens. For example, standard IVF treatment protocols includes a long phase of hormone stimulation of the female patient, e.g. 30 days. This protocol is initiated by suppressing the patient's own FSH and LH by gonadotropin releasing hormone GnRH or an analog of GnRH, and is followed by injections of exogenous gonadotropins, e.g. FSH and/or LH, in order to ensure development of multiple preovulatory follicles. At an appropriate stage of follicular growth, multiple oocytes are harvested by aspiration immediately before ovulation. The aspirated oocyte is subsequently fertilized *in vitro* and cultured, typically for three days before transferral of the resulting embryo into the uterus at the 4-8 cell stage.

Despite the fact that protocols such as those described above have been used in clinical protocols for a number of years, these protocols are not without some significant disadvantages. FSH has a relatively short half life and its use in the treatment of infertility generally requires daily administration, such daily administration has the related risk of producing ovarian hyper stimulation syndrome (OHSS), which in severe cases may be life-threatening. There are other additional side-effects from the gonadotropin preparations including weight gain, bloating, nausea, vomiting, the time involved with the monitoring process, and the unknown long-term cancer risk. These hormone treatment regimens will become even more of a problem when IVF is offered to perfectly normal women in these programs due to infertility problems associated with the males partner's poor sperm quality.

Due to the risks involved with administration of gonadotropins, various alternative protocols have been suggested. One way to alleviate the risks, side effects, and economic disadvantages of controlled ovarian stimulation protocols involves the retrieval of immature oocytes followed by *in vitro* maturation. In this approach, the female is without stimulation, or receives only minimal stimulation, and the retrieved oocytes are subjected to hormonal treatment *in vitro*. This *in vitro* maturation (IVM) protocol involves a significant reduction/elimination in a number of the side effects mentioned above and has the secondary economic advantages of reducing the

amounts of hormones used for the treatment. However, while in animals *in vitro* maturation (IVM) has become an efficient method for producing oocytes for IVF, the recorded success rates for clinical human IVM have been low (Cha, Trounson, Barnes, Russel).

Thus, there are problems associated with the current protocols used in oocyte generation for IVF. There remains a need for the production of greater quantities of oocytes that are amenable to ovulation.

SUMMARY OF THE INVENTION

The present invention is directed to methods of increasing the maturation of follicles in a female mammal in order to increase the production of oocytes in said mammal.

In certain aspects, the present invention provides a method of increasing follicle maturation comprising treating a mammal with a phosphodiesterase (PDE) inhibitor in an amount effective to stimulate follicular growth and maturation. Also included as an aspect of the invention are methods of increasing follicle maturation comprising treating a mammal with a PDE inhibitor and a gonadotropin hormone, wherein the PDE and gonadotropin are provided in a collective amount effective to stimulate follicular growth and maturation.

In another aspect, the invention provides a method of *in* vitro oocyte maturation, comprising treating an oocyte *in vitro* with a PDE inhibitor in an amount sufficient to cause oocyte maturation.

In yet another aspect, the invention provides a method of *in vitro* oocyte maturation comprising treating an oocyte *in vitro* with a PDE inhibitor and a gonadotropin hormone, wherein the PDE and gonadotropin are provided in a collective amount sufficient to cause oocyte maturation. Preferably, the increase in the number of mature oocytes is in the order of zero to one oocytes in the absence of said treatment being increased to four or more oocytes by the use of the methods described herein. Preferably the immature oocytes are located *in vivo* in a mammal. However, other embodiments contemplate the in vitro maturation of the immature oocytes.

A particular aspect of the present invention provides a method of increasing the follicle maturation in an animal comprising administering to the animal a composition comprising at least one PDE inhibitor and a gonadotropin hormone, wherein the PDE and gonadotropin are administered in a collective amount effective to increase the number of human chorionic gonadotropin responsive oocytes. In particularly preferred embodiments, the PDE inhibitor is a PDE-4 inhibitor. Exemplary PDE-4 inhibitors that may be used include but are not limited to is selected from the group consisting of Piclamilast, Roflumilast, Ariflo, Filaminast, Mesopram, D4418, Aroflylline, and CL1044, additional PDE-4 inhibitors may be used. Many are exemplified herein below, however it should be understood that analogs and derivatives of these compounds that have PDE-4 inhibitory activity also may be used. It is contemplated that the methods of the invention may be performed using only one PDE inhibitor. Alternative a cocktail of multiple PDE inhibitors may be employed. Such a cocktail may include one or more PDE-4 inhibitors in addition to one or more other PDE inhibitors. It is particularly contemplated that the composition may comprise at least one PDE-4 inhibitor and at least one other PDE inhibitor selected from the group consisting of a PDE-1 inhibitor, a PDE-5 inhibitor, a PDE-6 inhibitor, PDE-7 inhibitor, PDE-9 inhibitor, PDE-10 inhibitor, and PDE-11 inhibitor. In specific embodiments, the methods of the invention employ compositions which comprise two or more PDE-4 inhibitors.

In the methods of the present invention, the gonadotropin used for stimulating or increasing follicle maturation is selected from the group consisting of follicle stimulating hormone (FSH), luteinizing hormone (LH), and chorionic gonadotropin (CG). Preferably, the gonadotropin is FSH. In other embodiments, the method comprises administering FSH and further administering a non-FSH gonadotropin hormone, such as e.g., LH. Alternatively, the method comprises administering FSH in combination with a stimulator, agonist or adjuvant of FSH. Alternatively, the method may comprise administering a stimulator, agonist or adjuvant of FSH alone in combination with a PDE-4 inhibitor. Exemplary stimulators of FSH activity include aromatase inhibitors such as Letrozole, Anastrozole and Vorozole (see PCT/EP01/14730 and U.S. Patent Publication No. 20020103106).

In specific embodiments, the PDE inhibitor and the gonadotropin hormone treatment are administered concurrently. By "gonadotropin hormone

treatment" the present invention contemplates FSH treatment alone, FSH treatment in combination with a non-FSH gonadotropin, or treatment with a stimulant or other agonist of FSH activity either alone or in combination with FSH and/or other non-FSH gonadotropin. In some embodiments, the PDE inhibitor is administered prior to the administration of the gonadotropin hormone. In other embodiments, the PDE inhibitor is administered after the administration of the gonadotropin hormone. The dosage of the FSH may be any dosage routine used in a clinical setting. For example, the FSH may be administered at a dosage range of from about 5 to 450 IU/day. More preferably, the FSH is administered at a dosage range of from about 5 to 75 IU/day. In specific embodiments, it is contemplated that the administration of the PDE-4 or other PDE inhibitor will allow a reduction in the amount of FSH normally administered to an individual. The FSH may be recombinant FSH (r-FSH), preferably, human recombinant FSH (hFSH). In other embodiments, the FSH is purified from urine.

The invention also provides a method of increasing the *in vivo* development of preovulatory follicles in a mammal, the method comprising administering to the mammal a composition comprising at least one PDE-4 inhibitor and an exogenous FSH hormone. Such a method may further comprise suppression of endogenous FSH and LH production in the mammal prior to administration of the PDE-4 inhibitor and the FSH hormone. Preferably, the exogenous FSH hormone is a recombinant FSH hormone. In other embodiments, the exogenous FSH hormone is urinary FSH hormone.

In specific embodiments the PDE-4 inhibitor is administered in a dose of from about 0.05 mg/day to about 5 mg/day. It is contemplated that the dosage of the inhibitor will vary according to the specific inhibitor as well as the characteristics of the patient being treated. IT is contemplated that the dosage may be 0.05 mg/day, 0.075 mg/day, 0.10 mg/day; 0.125 mg/day; 0.150 mg/day; 0.175 mg/day; 0.20 mg/day; 0.225 mg/day; 0.250 mg/day; 0.275 mg/day; 0.30 mg/day; 0.325 mg/day; 0.350 mg/day; 0.375 mg/day; 0.40 mg/day; 0.425 mg/day; 0.450 mg/day; 0.475 mg/day; 0.50 mg/day; 0.525 mg/day; 0.550 mg/day; 0.575 mg/day; 0.60 mg/day; 0.625 mg/day; 0.650 mg/day; 0.675 mg/day; 0.70 mg/day; 0.725 mg/day; 0.750 mg/day; 0.775 mg/day; 0.80 mg/day; 0.825 mg/day; 0.850 mg/day; 0.875 mg/day; 0.90 mg/day; 0.925 mg/day; 0.950 mg/day; 0.975 mg/day; 1.0 mg/day; 1.25 mg/day;

1.5 mg/day; 1.75 mg/day; 2.0 mg/day; 2.25 mg/day; 2.5 mg/day; 2.75 mg/day; 3.0 mg/day; 3.25 mg/day; 3.5 mg/day; 3.75 mg/day; 4.0 mg/day; 4.25 mg/day; 4.5 mg/day; 4.75 mg/day; 5.0 mg/day or more per day. In preferred embodiments, it is contemplated that the PDE-4 inhibitor is administered as in a dosage of from about 10 mg/day to about 200 mg/day. Those of skill in the art would understand that these are merely exemplary dosage amounts and ranges. It should be understood that any individual numerical amount between the dosages expressly recited herein is particularly contemplated and each of the individual values between these doses is specifically intended to be within the scope of the invention. The individual values between the specific doses recited herein have been omitted simply for ease of legibility and not because it was intended to be excluded from the scope of the application. The same is true of the other, non-PDE inhibitor compositions administered herein.

In other embodiments, it is contemplated that the FSH is administered in a dosage range of 5 IU FSH/day to 75 IU FSH per day. More particularly, the FSH may be administered as a dosage of 150 IU FSH per day. The FSH is preferably administered in a single dose. Other embodiments contemplate administering the FSH in multiple doses. The FSH may be administered intramuscularly, subcutaneously or via any other convenient method of administration. Preferably, the FSH is administered between day 2 and day 14 of the menstrual cycle of the mammal. In specific embodiments, the FSH is administered for between 7 to 12 consecutive days.

In particular embodiments, the suppression of endogenous FSH and LH comprises administering gonadotropin releasing hormone (GnRH) or an analog thereof to the mammal. More particularly, the GnRH is administered to the mammal for 30 days prior to administration of the PDE-4 inhibitor and the FSH hormone. The dosage amounts of the GnRH may be any dosage routinely employed for suppression of endogenous gonadotropins. Such embodiments may typically employ GnRH or an antagonist thereof administered in a dosage range of from about 0.25 mg to about 3 mg GnRH on a daily basis.

In the methods of the present invention, the administration of PDE-4 inhibitor and FSH produces more hCG ovulatable oocytes in the mammal as compared to the production of hCG ovulatable oocytes in the absence of the

administration of the PDE-4 inhibitor and FSH. For example, in the absence of such treatment the subject may produce zero or one hCG ovulatable oocytes, however the treatment increases that number to four or more hCG ovulatable oocytes. Thus, there may be produced 4, 5, 6, 7, 8 9 10 or more oocytes that are harvestable as a result of the methods of the present invention. The methods of the invention contemplate harvesting the oocytes 12 days after the initial administration of the PDE-4 inhibitor and the FSH hormone. The methods may further involve fertilizing the harvested oocytes *in vitro*, and culturing the harvested, fertilized oocytes to the 4-8 cell stage. Such 4-8 cell stage fertilized oocytes may further be transferred to the uterus of a mammal. The mammal may be same mammal from which the oocytes were harvested or it may be a different mammal from which the oocytes were harvested.

The invention further provides a kit for the treatment of infertility, the kit comprising a first composition comprising at least one PDE-4 inhibitor in a pharmaceutically acceptable formulation, and a second composition comprising FSH in a pharmaceutically acceptable formulation. The kit may comprise urinary FSH or recombinant FSH. In either case the FSH is preferably human FSH. The FSH of the kit is preferably provided in a unit dose of between about 5 IU FSH and about 75 IU FSH. The kit may further comprise a third composition comprising LH in a pharmaceutically acceptable formulation. Exemplary amounts of LH employed in the unit doses in the kits are doses of between about 75 IU LH and about 150 IU LH.

Other features and advantages of the invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, because various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE FIGURES

The following drawings form part of the present specification and are included to further illustrate aspects of the present invention. The invention may be better understood by reference to the drawings in combination with the detailed description of the specific embodiments presented herein.

FIG. 1A-1C *In vitro* studies to determine the ability of PDE-4 inhibitors to induce cAMP in rat granulosa cells and/or human FSH receptor-expressing porcine granulosa cells.

FIG. 2. *In vivo* demonstration of induction of follicle maturation by administration of a combination of a low dose of FSH with varying concentrations (0.08mg/kg; 0.4 mg/kg; 2 mg/kg) of Piclamilast.

FIG. 3. *In vivo* demonstration of induction of follicle maturation by administration of a combination of a low dose of FSH with varying concentrations (0.08mg/kg; 0.4 mg/kg; 2 mg/kg) of Roflumilast.

FIG. 4. *In vivo* demonstration of induction of follicle maturation. Comparison of oocyte production upon stimulation using low and high doses of FSH alone; varying concentrations (0.08mg/kg; 0.4 mg/kg; 2 mg/kg) of Piclamilast and using a combination of a low dose of FSH with varying concentrations (0.08mg/kg; 0.4 mg/kg; 2 mg/kg) of Piclamilast. Results from two independent studies are shown.

FIG. 5. *In vivo* demonstration of induction of follicle maturation. Cumulative data demonstrating increased oocyte production upon stimulation using a combination of a low dose of FSH with varying concentrations (0.08mg/kg; 0.4 mg/kg; 2 mg/kg) of Piclamilast.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Ovarian stimulation using gonadotropin hormones is now recognized as a significant treatment regimen for couples have suboptimal levels of fertility. IVF procedures rely on the use of significant quantities of FSH and other gonadotropin hormones in order to stimulate the production of oocytes for fertilization. However, such ovarian stimulation often leads to significant deleterious side effects. Therefore, there is a need for additional methods of increasing the production of ovulatable oocytes that do not depend solely on ovarian stimulation with gonadotropins.

The present invention is directed to inducing follicle maturation using phosphodiesterase (PDE) inhibition. It has been discovered that inhibitors of PDE, when administered to immature rats, lead to an increase in the number of hCG-ovulatable oocytes. More particularly, PDE-4 inhibitors when administered in

combination with suboptimal doses of FSH produce substantial increases in the number of such oocytes.

In specific exemplary embodiments, it was discovered that administration of PDE-4 inhibitors, Piclamilast and Roflumilast, increased FSH-induced follicle maturation. The most dramatic effects are seen when low FSH is administered in combination with a PDE-4 inhibitor. As can be seen from the data in Figures 3-5, administration of a low dose of FSH alone will produce *e.g.*, 1 ovulated ovum per rat (see FIG 2 and FIG.3), whereas ovarian stimulation with a high dose of FSH will typically yield in the order of 15-18 ovulated ova per rat. The problems of using high doses of FSH have been discussed above. The present invention demonstrates that the number of ovulatable oocytes can be markedly increased from the basal numbers seen with low FSH when the rats are treated with PDE-4 inhibitors. Indeed, as can be seen from FIG. 2 and FIG. 3, the combined administration of low dose of FSH with 0.4 mg/kg of either Piclamilast or Roflumilast produces more ovulatable ova than even the high dose of FSH alone (FIG. 2 and FIG. 3). In addition, as can be seen from FIG. 5 it also was noted that higher doses of Piclamilast (e.g., 2mg/kg) alone, without co-administration of FSH, increased the hCG ovulatable ova.

Given the above exemplary data, it is contemplated that PDE inhibitors, and particularly PDE-4 inhibitors, may be used in producing ovulatable oocytes *in vivo*. This will be particularly useful in the context of the treatment of female infertility, but even a normal female will benefit from such treatment, particularly if there is a desire to increase the likelihood of pregnancy. In more specific embodiments, PDE-4 inhibitors are contemplated to be particularly useful in enhancing or increasing follicle maturation by producing an increase in the number of ovulatable oocytes in mammals *in vivo* as compared to the number of ovulatable oocytes that are produced in the absence of administration of the PDE-4 inhibitors.

Given the findings in FIG. 4 and FIG. 5, it is contemplated that administration of PDE-4 inhibitors alone, without concomitant stimulation with low doses of FSH will be sufficient to increase the ovulatable oocytes in a female. As such, certain methods of the invention entirely circumvent the need for ovarian stimulation with exogenous FSH. Alternatively, the findings shown in Figures 2-5, the methods of the invention also comprise administering low levels of FSH in combination with PDE-4 inhibitors is sufficient to produce a marked increase in

ovulatable oocytes. Regardless of whether the PDE-4 inhibitors are administered alone or as part of a combination treatment with low levels of FSH, the methods of the present invention provide a significant advance in the art by markedly reducing the amount of FSH stimulation required to yield a therapeutically beneficial increase in the ovulatable oocytes. Such a marked reduction in the amount of FSH needed is of great benefit because it reduces or eliminates many of the problems associated with administration of gonadotropins in fertility treatments. Principal among these being a decreased likelihood of inducing OHSS in female undergoing fertility treatment.

While much of the discussion presented herein is in terms of the use of PDE-4 inhibitors, the findings of the present invention support the use of other PDE inhibitors either alone, in combination with PDE-4 inhibitors, and or in combination with low doses of FSH. For example, it is contemplated that other PDE inhibitors e.g., a PDE-5 inhibitor may be used in a method to increase the number of ovulatable oocytes in a mammal. Such a method may use the PDE-5 inhibitor alone or alternatively, the method may use the PDE-5 inhibitor in combination with either a PDE-4 inhibitor or a low dose of FSH. Yet another preferred alternative is a method in which a PDE-4 inhibitor is administered in combination with the PDE-5 inhibitor and a low dose of FSH. It is contemplated that the in such methods, the dose of FSH used when a PDE inhibitor is administered may be less than or equal to 75% of the dose of FSH that would be required in the same patient in the absence of the use of the PDE inhibitor in order to achieve the same level of follicular maturation. More preferably, the amount of FSH will be less than or equal to about 50% of the dose of FSH that would be required in the same patient, even more preferably, the dose will be less than or equal to at least 30% of the dose of FSH that would be required in the same patient without the PDE inhibitor. The combinations and methods of using the PDE inhibitors and FSH compoisitions are discussed in further detail herein below.

A. FSH and other Gonadotropin Compositions

FSH is a pituitary glycoprotein hormone that is composed of two subunits. The α-subunit is common to FSH as well as the other glycoproteins, LH, hCG and TSH, the β-subunit confers FSH specificity. The field of infertility treatment is advanced and there are presently numerous FSH preparations that are commercially available and may be used in the methods of the invention. Such

commercial preparations include urinary-derived FSH compositions and recombinant FSH compositions. These compositions include, *e.g.*, Pergonal™, Fertinex™, Repronex™, Bravelle™, Humegon™, Gonal-F, Follistim™. These are merely exemplary commercial FSH preparations and those of skill in the art will understand that it may be possible to produce other such FSH preparations for use in the methods and compositions of the present invention. To the extent that the preceding compositions provide exemplary guidance as to formulations and dosages of FSH that may be used, they are discussed in further detail below. However, it should be understood that such doses and formulations may readily be modified and still be useful in the context of the present invention as long as the FSH dosages and formulations when administered in combination with one or more PDE inhibitors produce an increase in the number of ovulatable oocytes *in vivo* as compared to the number of oocytes produced in the absence of such administration.

Humegon™ (Organon, West Orange, NJ) is a purified preparation of gonadotropins that contains FSH and LH activity. The properties, indications and protocols for the use of this preparation of gonadotropins is discussed in detail in the Physician's Desk Reference. (see e.g., PDR™, 52nd Edn. 1998, pages 1949-1951). Briefly, each vial contains 75 IU or 150 IU FSH, 75 IU or 150 IU LH. Humegon™ and hCG may be administered in a sequential manner for ovulation and pregnancy in anovulatory infertile women in whom the cause of anovulation is functional and is not due to ovarian failure. Similarly Humegon™ and hCG are indicated for stimulating the development of multiple follicles (*i.e.*, stimulation of follicle development) for ovulatory patients participating in an *in vitro* fertilization program. Typically, the dose of Humegon™ used to produce follicle maturation needs to be adjusted and individualized for each patient. However, typically the initial recommended dose would be 75 IU FSH/LH/day administered intramuscularly for 7 to 12 days followed by a dose of hCG (5000 U to 10000 U), administered one day after the last dose of Humegon™. Typically, the Humegon™ therapy should not continue for more than 12 consecutive days in a single course of therapy. For administration, the vial of Humegon™ (containing either 75 IU FSH/LH or 150 FSH/LH) is dissolved in 2 ml sterile saline and immediately administered intramuscularly.

Pergonal™ (Serono Laboratories Inc., Randolph, MA), described in the Physician's Desk Reference (see e.g., PDR™, 52nd Edn. 1998, pages 2773-2775) is another commercially described purified preparation of gonadotropins prepared

from the urine of postmenopausal women that may be used to supply the FSH compositions of the methods of the present invention. This composition is formulated for intramuscular administration. Again, this is a pharmaceutical composition that comprises unit doses of 75 IU FSH/LH or 150 IU FSH/LH. This pharmaceutical agent is well-recognized as a composition for administration to women for the production of follicular growth in women who do not have ovarian failure. To effect ovulation, rather than just follicle maturation, the 7-12 day course of Pergonal™ administration is followed by a bolus of hCG administration.

Repronex™ (Ferring Pharmaceutical Inc., Tarrytown, NJ), described in the Physician's Desk Reference (see e.g., PDR™, 57th Edn. 2003, pages 1325-1327) is another exemplary purified preparation of gonadotropins isolated from the urine of post-menopausal women. This composition, available in unit doses of 75 IU or 150 IU FSH/LH activity, is administered for 7 to 12 days to produce ovarian follicular growth. Once sufficient follicular maturation has occurred, hCG is administered to induce ovulation. Repronex™ may be administered subcutaneously or intramuscularly to infertile patients with oligoanovulation or for patients undergoing assisted reproductive therapy. In the former indication, for patients that have received therapeutic intervention for the suppression of endogenous gonadotropin, Repronex™ is administered in an initial dose of 150 IU daily for the first five days of treatment. Based on clinical monitoring of e.g., serum estradiol levels and vaginal ultrasound monitoring, subsequent dosing may be adjusted up or down according to the patient's individualized response. Preferably, the dosage adjustment should not be made more than one every other day and should not exceed a change of more than 75 to 150 IU per adjustment. Preferably the maximum daily dose should not exceed 450 IU and the maximum number of days in consecutive course of Repronex™ therapy should not exceed 12 days. Such dosage adjustment guidelines are applicable to other FSH preparations discussed herein. If the patient appears to show the signs of follicle maturation, hCG is administered 1 day after the cessation of the Repronex™-based therapy.

In patients that are being treated with Repronex™ for assisted reproductive therapy, where the patient has received an initial gonadotropin suppressive therapy (e.g., GnRH or antagonist pituitary suppression) the typical initial dose may be 225 IU Repronex™, which may subsequently be adjusted according to the patient's individual response. Once a sufficient follicular development is evident,

hCG (5000-10000 USP units) is administered to induce final follicular maturation in preparation for oocyte retrieval. In the event that the ovaries are abnormally enlarged as a result of Repronex™ administration hCG is withheld in order to decrease the possibility of developing OHSS. As indicated above, this composition is administrable subcutaneously. For subcutaneous administration, the Repronex™ is mixed 2ml with saline and the subcutaneous injection is delivered to the lower abdomen.

Fertinex™ (Serono Laboratories Inc., Randolph, MA), described in the Physician's Desk Reference (see e.g., PDR™, 52nd Edn. 1998, pages 2771-2773) is highly purified FSH preparation purified from the urine of postmenopausal women. The purification of FSH composition is achieved through immunoaffinity chromatography using a murine monoclonal antibody to FSH, and produces an FSH with a specific activity profile of from about 8,500-13500 IU FSH/mg protein, very low amounts of LH, and a greater than 95% purity with respect to other urinary proteins. Such purification methods can readily be used to obtain FSH from non-commercial sources, e.g., urine of post-menopausal women. Fertinex™ is subcutaneously administrable and is supplied in containers containing 75 IU FSH or 150 IU FSH. This FSH has been administered in exemplary dosage ranges varying from 75 IU to 300 IU/day.

Bravelle™ (Ferring Pharmaceutical Inc., Tarrytown, NJ), described in the Physician's Desk Reference (see e.g., PDR™, 57th Edn. 2003, pages 1325-1327) is another exemplary highly purified FSH preparation that may be used in the present methods. Bravelle™ may be administered subcutaneously or intramuscularly and is available in unit doses 75 IU and 150 IU FSH.

Gonal F™ is a recombinant FSH preparation suitable for subcutaneous administration. The properties and characteristics of Gonal F™ are described in detail in the Physician's Desk Reference (PDR™, 57th Edn. 2003, pages 3124-3128).

In addition to these commercially available compositions, those of skill in the art may chose to purify FSH from natural source, e.g., urine of post-menopausal women, using techniques well known to those of skill in the art (See e.g., U.S. Patent No. 5,767,067).

Alternatively, those of skill in the art may choose to produce recombinant FSH using techniques well known to those of skill in the art. It is particularly contemplated that long-lasting FSH agonists would be useful in the

methods of the invention. For example, it is known that hCG has a longer half life than FSH. Both of these gonadotropins share a common α-subunit, with the specific activity being conferred by the β-subunit. It has previously been demonstrated that the α-subunit of one gonadotropin may be used with the β-subunit of another and still yield a physiologically active chimeric gonadotropin. Further it has been demonstrated that the increased biopotency of hCG as compared to LH was due to the carboxy-terminal peptide of the β-subunit of hCG (Matzuk et al., Endocrinology 126:376-383, 1990). Long lasting agonists of FSH may be generated which contain a carboxy-terminal peptide extension of hCG β-subunit at the carboxy terminus of the FSH b subunit. (LaPolt et al., Endocrinology, 131:6, 2514-2520, 1992). Such chimeric molecules have been shown to possess a markedly increased circulating half life and potency as compared to wild-type FSH (Fares et al., Proc. Nat'l Acad. Sci., 89:4304-4308, 1992).

Regardless of the source of FSH, it has been demonstrated herein that significant induction of follicle maturation can be achieved using suboptimal doses of FSH. As discussed above, the typical dosage of FSH administered in fertility treatment protocols ranges from about 75 IU FSH/day to about 450 IU FSH/day for a course of from about 7 days to about 12 days. In the methods of the present invention, it is contemplated that the dose of FSH used for stimulating follicular maturation in combination with the PDE-4 or other PDE inhibitors may be the same as the doses presently being used for treatment of oligoanovulation and/or in assisted reproductive technologies (*i.e.*, vary between 75 IU FSH to about 450 IU FSH per day). However, given that the present invention teaches that it is possible to obtain follicular maturation even with suboptimal doses of FSH, when such doses are administered in combination with a PDE-4 inhibitor, it is preferred that the dose be lower than these typical doses.

It is contemplated that the methods of the present invention may use as little as 5 IU FSH/day. Thus it is contemplated that any given treatment regimen may employ 5 IU FSH/day, 10 IU FSH/day, 15 IU FSH/day, 20 IU FSH/day, 25 IU FSH/day, 30 IU FSH/day, 35 IU FSH/day, 40 IU FSH/day, 45 IU FSH/day, 50 IU FSH/day, 55 IU FSH/day, 60 IU FSH/day, 65 IU FSH/day, 70 IU FSH/day, 75 IU FSH/day, or more units of FSH per day. Of course, it should be understood that these are merely exemplary daily dosages and other doses of integers between any of the specifically recited doses also may be used in the treatment methods of the invention.

Further, it should be understood that the dosage may be adjusted up or down during any given course of FSH administration. The FSH may be administered through any route normally employed for the administration of gonadotropin hormones. Most preferably the administration is either via intramuscular or subcutaneous injection. Throughout the treatment protocols, the patient is monitored for signs of adverse reaction including for signs of OHSS.

In addition to FSH, other gonadotropin hormones will be used in the methods of the present invention and packaged in the kits described herein. Such hormones include hCG. This is commercially available as Novarel™ (Ferring Pharmaceutical Inc., Tarrytown, NJ), described in the Physician's Desk Reference (see e.g., PDR™, 57th Edn. 2003, pages 1324-1325) and is a gonadotropin produced by the human placenta and obtained from the urine of pregnant women. Another commercial preparation of hCG is Pregnyl™ (Organon, West Orange, NJ). The properties, indications and protocols for the use of this hormone are discussed in detail in the Physician's Desk Reference. (see e.g., PDR™, 57th Edn. 2003, pages 2401). Both of these preparations are for intramuscular administration. Typically, this hormone is administered in a dosage of between about 5000 Units and 10 000 Units to induce ovulation.

Yet another hormone that may be used and packaged herein is GnRH. There are numerous commercial sources of this hormone. GnRH and analogs thereof are commercially available as Cetrotide™ (Serono; see PDR™, 57th Edn. 2003, pages 3119-3121); Eligard™ (Sanofi-Synthelabo, PDR™, 57th Edn. 2003, page 2994); Lupron™ (PDR™, 57th Edn. 2003, page 3185); and Zoladex™ (AstraZeneca PDR™, 57th Edn. 2003, page 695). These agents are used to suppress LH/FSH production in women and are therefore used to delay ovulation. Typical doses of these agents vary from about 0.25 mg to about 3 mg. Ovarian stimulation therapy with FSH is typically initiated on the 2nd or 3rd day of the menstrual cycle. The GnRH or analogs thereof are administered either once daily (lower dose, e.g., 0.25 mg), or as a single dose (e.g., 3 mg) during the early to mid follicular phase. GnRH is administered up until the day of hCG administration. When ultrasound analyses reveal that the follicles are of an adequate size, hCG is administered to induce ovulation and final maturation of the oocyte.

B. Phosphodiesterase Inhibitors

PDE are a family of enzymes responsible for the metabolism of the intracellular second messengers cyclic AMP (cAMP) and cyclic GMP (cGMP). PDE-4 is a cAMP specific PDE that is the major, if not sole, cAMP metabolizing enzyme found in inflammatory and immune cells, and contributes significantly to cyclic AMP metabolism in smooth muscles. PDE-4 is inhibited by the antidepressant Rolipram (4-[3-(Cyclopentyloxy)-4-methoxy-phenyl]-2-pyrrolidinone; A.G. Scientific, Inc., San Diego, CA). Rolipram was the first generation of PDE-4 inhibitors developed (see Conti, Biology of Reproduction 67:1653-1661, 2002). Subsequently, other such inhibitors have been identified, including but not limited to Piclamilast, Roflumilast, Ariflo, Filaminast, Mesopram, D4418, Aroflylline, CL1044. In addition, other PDE inhibitors such as Sildenafil, AS701948/1 and AS701947/1 also will be useful in the present invention. Thus, particularly preferred PDE-4 inhibitors for use in the present invention include lirimilast. (Bayer AG); CDP-840 (Celltech Group PLC), NCS-613 (Centre National de la Recherche Scientifique (CNRS) E-4021(Eisai Co Ltd), GRC-3785 (Glenmark Pharmaceuticals Ltd), IC-485 (ICOS Corp); IPL-455903 (Inflazyme Pharmaceuticals Ltd), ONO-6126 (Ono Pharmaceutical Co Ltd), Tofimilast (Pfizer Inc.), Piclamilast (Rhone-Poulenc SA (Aventis SA)), Cilomilast. (SmithKline Beecham PLC), Filaminast. (Wyeth-Ayerst Pharmaceuticals Inc), WAY-126120 (Wyeth-Ayerst Pharmaceuticals Inc0), Mesopram (Schering), and Roflumilast (Altana).

The above and other PDE-4 inhibitors are well known to those of skill in the art and have been described in *e.g.*, U.S. Patent No. 6649633; U.S. Patent No. 6624181; U.S. Patent No. 6127363; DE 1545687, DE 2028869, DE 2123328, DE 2315801, DE 2402908, DE 2413935, DE 3900233, EP 0103497, EP 0139464, EP 0158380, EP 0163965, EP 0335386, EP 0389282, EP 0428302, EP 0435811, EP 0459505, EP 0470805, EP 0490823, EP 0506194, EP 0511865, EP 0527117, EP 0393500, EP 0510562, EP 0553174, EP 0557016, EP 0626939, EP 0664289, EP 0671389, EP 0685474, EP 0685475, EP 0685479, EP 0736532, EP 0738715, EP 0748805, EP 0763534, EP 0816357, EP 0819688, EP 0819689, EP 0832886, EP 0834508, EP 0848000, JP 92234389, JP 94329652, JP 95010875, JP 98072415, JP 98147585, U.S. Pat. Nos. 5,703,098, 5,739,144, WO 9117991, WO 9200968, WO 9212961, WO 9307146, WO 9315044, WO 9315045, WO 9318024, WO 9319068, WO 9319720, WO 9319747, WO 9319749, WO 9319751, WO 9325517, WO

9402465, WO 9412461, WO 9420455, WO 9422852, WO 9427947, WO 9501338, WO 9501980, WO 9503794, WO 9504045, WO 9504046, WO 9505386, WO 9508534, WO 9509623, WO 9509624, WO 9509627, WO 9509836, WO 9514667, WO 9514680, WO 9514681, WO 9517392, WO 9517399, WO 9519362, WO 9520578, WO 9522520, WO 9524381, WO 9527692, WO 9535281, WO 9535283, WO 9535284, WO 9600218, WO 9601825, WO 9606843, WO 9611690, WO 9611917, WO 9612720, WO 9631486, WO 9631487, WO 9635683, WO 9636595, WO 9636596, WO 9636611, WO 9636625, WO 9636638, WO 9638150, WO 9639408, WO 9640636, WO 9703967, WO 9704779, WO 9705105, WO 9708143, WO 9709345, WO 9712895, WO 9718208, WO 9719078, WO 9720833, WO 9722585, WO 9722586, WO 9723457, WO 9723460, WO 9723461, WO 9724117, WO 9724355, WO 9725312, WO 9728131, WO 9730999, WO 9731000, WO 9732853, WO 9735854, WO 9736905, WO 9743288, WO 9744036, WO 9744322, WO 9747604, WO 9748697, WO 9804534, WO 9805327, WO 9806692, WO 9806704, WO 9807715, WO 9808828, WO 9808830, WO 9808841, WO 9808844, WO 9809946, WO 9809961, WO 9811113, WO 9814448, WO 9818796, WO 9821208, WO 9822453, WO 9845268, WO 9855481, WO 9856756, WO 9905111, WO 9905112, WO 9505113, WO 9906404, WO 9918095, WO 9501338, WO 9603399, WO 9636625, WO 9636626, WO 9735854, WO 9821208, WO 9831674, WO 9840382, WO 9855481, WO 9905111, WO 9905112, WO 9905113, WO 9931071 and WO 9931090. Any of these substances may be used as the PDE-4 inhibitor composition in the context of the present invention. Substances that have good oral availability are particularly preferred.

These inhibitors of PDE-4 may be administered through any route commonly employed for the administration of a PDE inhibitor. Typically these chemical agents are formulated for oral administration. The tablets, may be formulated to comprise 50 μg; 100 μg; 200 μg; 250 μg; 300 μg; 400 μg; 450 μg; 500 μg. Currently, roflumilast is being developed for pulmonary indications with doses as of about 400 ug/day, for oral administration. It is contemplated that such doses may also be useful in the context of the present invention. The PDE-4 inhibitors are administered to the subject in a daily dose of 200 μg/day; 300 μg/day; 400 μg/day; 500 μg/day or even as much as 1 to 5 mg/day. Typically, the patient may receive as little as 100 μg/day for the course of treatment. Of course it should be understood the subject may receive more or less of the PDE-4 inhibitor according to individualized

requirement. Typically, doses greater than 100 mg/day should be avoided. The PDE-4 inhibitor may be delivered in a single dose or alternatively may be subdivided and administered in multiple doses over a given period of time. Administration of ordinary tablets containing the inhibitors may be once, twice, three or more times a day. Also it should be understood that while oral administration is preferred, similar doses may be administered through other routine routes of administration.

Sildenafil is a pharmaceutically approved PDE-5 inhibitor that may provide additional guidance as to the formulations and routes of administration of PDE-4 inhibitors (see PDR™ 57th Edn. Pages 2653-2656.) For treatment protocols, those of skill may use the guidelines used for this pharmaceutical PDE inhibitor. Other PDE-5, as well as PDE-1 inhibitors may be useful in the present invention. Such inhibitors have been described in U.S. Patent Application No. 60/470,434 entitled Ihibitors of PDE enzymes in infertility," U.S. Patent Application No. 10/014, 812 (U.S. Patent Publication No. 20020103106, incorporated herein by reference in its entirety) entitled "Methods of Inducing Ovulation" which describes a variety of PDE3/4 inhibitors for triggering ovulation that may be used in the follicle maturation methods of the present invention. It should be noted that the methods of the present invention are directed to methods of increasing follicle maturation and are distinct from methods of inducing ovulation. Additional compositions and are described in PCT/EP01/14730.

Exemplary inhibitors that may be useful in the combined therapies discussed herein include, but are not limited to, 5-[2-ethoxy-5-(4-methyl-1 -piperazinylsulphonyl)phenyl]-1 -methyl-3-n-propyl-1,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one (sildenafil); 5-(2-ethoxy-5-morpholinoacetylphenyl)-1 -methyl-3-n-propyl-1,6-dihydro-7H-20 pyrazolo[4,3-d]pyrimidin-7-one; 3-ethyl-5-[5-(4-ethylpiperazin- 1 -ylsulphonyl)-2-n-propoxyphenyl]-2-(pyr- idin-2-yl) methyl-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one; 3-ethyl-5-[5-(4-ethylpiperazin- 1 -ylsulphonyl)-2-(2-methoxyethoxy)pyridi- n-3-yl]-2-(pyridin-2-yl) methyl-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one; (+)-3-ethyl-5-[5-(4-ethylpiperazin-1-ylsulphonyl)-2-(2-methoxy-1(R)-methylethoxy)pyridin-3-yl]-2-methyl-2,6-dihydro-7 H-pyrazolo[4 ,3-d] pyrimidin-7-one; 5-[2-ethoxy-5-(4-ethylpiperazin-1-ylsulphonyl)pyridin-3-yl]-3-ethyl-2-[2-methoxyethyl]-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one; 5-[2-iso-butoxy-5-(4-ethylpiperazin-1-ylsulphonyl)pyridin-3-yl]-3-ethyl-2-(1-methylpiperidin-4-yl)-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one;

5-[2-ethoxy-5-(4-ethylpiperazin-1 -ylsulphonyl)pyridin-3-yl]-3-ethyl-2-phenyl-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one; 5-(5-acetyl-2-propoxy-3-pyridinyl)-3-ethyl-2-(1 -isopropyl-3-azetidinyl)-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one; 5-(5-acetyl-2-butoxy-3-pyridinyl)-3-ethyl-2-(1-ethyl-3-azetidinyl)-2 ,6-dihydro-7H-pyrazolo [4,3-d]pyrimidin-7-one; (6R, 12aR)-2,3,6,7,12,12a-hexahydro-2-methyl-6-(-3,4-methylenedioxyphenyl)pyrazino[2',1 ':6,1]pyrido[3,4-b]indole-1,4-dione (Tadalafil; IC-351), i.e. the compound of examples 78 and 95 of published international application W0 95/19978, as well as the compound of examples 1, 3, 7 and 8 therein; 2-[2-ethoxy-5-(4-ethyl-piperazin-1-yl-1-sulphonyl)-phenyl]-5-methyl-7-propyl-3H-imidazo [5,1 -f][1,2,4]triazin-4-one (vardenafil); the compound of example 11 of published international application W093/07124 (EISAI); compounds 3 and 14 from Rotella D P, J. Med. Chem., 2000, 43,1257; 4-bromo-5-(pyridylmethylamino)-6-[3-(4-chlorophenyl)-propoxy]-3(2H)pyridazinone; 1 -[4-[(1,3-benzodioxol-5-ylmethyl)amiono]-6-chloro-2-quinozolinyl]-4-piperidine-carboxylic acid, monosodium salt; (+)-cis-5,6a,7,9,9,9a-hexahydro-2-[4-(trifluoromethyl)-phenylmethyl-5-methyl-cyclopent-[4,5]imidazo[2,1-b]purin-4(3H)one; furaziocillin; cis-2-hexyl-5-methyl-3,4,5,6a,7,8,9,9a-octahydrocyclopent[4,5]-imidazo[2- ,1 -b]purin-4-one; 3-acetyl-1-(2-chlorobenzyl)-2-propylindole-6- carboxylate; 3-acetyl-1 -(2-chlorobenzyl)-2-propylindole-6-carboxylate; 4-bromo-5-(3-pyridylmethylamino)-6-(3-(4-chlorophenyl)propoxy)-3-(2H) pyridazinone; 1-methyl-5(5-morpholinoacetyl-2-n-propoxyphenyl)-3-n-propyl-1,6-dihydro- 7H-pyrazolo (4,3-d)pyrimidin-7-one; 1 -[4-[(1,3-benzodioxol-5-yl methyl)amino]-6-chloro-2-quinazolinyl]-4-piperidinecarboxylic acid, monosodium salt; Pharmaprojects No. 4516 (Glaxo Wellcome); Pharmaprojects No. 5051 (Bayer); Pharmaprojects No. 5064 (Kyowa Hakko; see WO 96/26940); Pharmaprojects No. 5069 (Schering Plough); GF-196960 (Glaxo Wellcome); E-8010 and E-4010 (Eisai); Bay-38-3045 & Bay-38-9456 (Bayer), Vinpocetine (Richter Gideon); SCH-51866 (Schering-Plough), SCH-59498; compounds no. 31, 33, 50 described in Ahn et al.; J. Med. Chem.; 1997, 40, 2196-2210, dipyridamole, AWD-12-171 and AWD-12-217 (ASTA Medica), BMS-341400 (Bristol Meyers Squibb), UK-343,664 (Pfizer), 5E-3623, 5E-3569, 5E-3657, E4021 (Eisai), KS 505a (Kyowa Hakko Kogyo), YC-1 (Yung Shin Pharmaceutical Industries), IDDB reference number 323951 (Bayer), WIN-61691 (Sanofi Winthrop), FR226807 (Fujisawa), IDDB references 461317, 462503, 461321, 461324, 466146 (Johnson & Johnson).].

Those of skill in the art are referred to U.S. Provisional Application Serial No. 60/470,434 entitled "inhibitors of PDE enzymes in infertility." In said specification are described methods and composition of using PDE1 and/or PDE5 inhibitors for inducing ovulation and controlled ovarian hyperstimulation for in vitro fertilization. The entire document is incorporated herein by reference in its entirety for its teaching of inhibitors and protocols for administering such inhibitors. Any of the inhibitors disclosed therein may be used in the protocols of the present invention.

Particularly preferred PDE4 inhibitors that may be used herein include but are not limited to Roflumilast (methods and compositions for making this compound may be found in WO9501338), Piclamilast (methods of making the same are described in J.Med. Chem. 1994, 37,1696-1703), Ariflo/Cilomilast (methods of making the same are described in J.Med. Chem. 1998, 41,821-835), Mesopram (methods of making the same are WO97/15561), Filaminast (methods of making the same are described in EP0470 805 B1).

C. Delivery of a Combination of FSH and Phosphodiesterase Inhibitors to Induce Follicle Maturation

In certain aspects, the methods of the invention contemplate the combined use of PDE-4 inhibitors with an FSH containing composition to increase follicle maturation. However, in addition to therapies based solely on the delivery of FSH/PDE-4 combination therapy, the methods of the present invention also contemplate combination therapy with a third composition that will enhance the follicle maturation effects of the treatment methods of the invention. Such a third composition may be a second PDE-4 inhibitor, or a second inhibitor of PDEs that is not specifically a PDE-4 inhibitor. For example, it is contemplated that the methods of the present invention may further involve administering a PDE-1 and/or a PDE-5 inhibitor. In addition, LH and/or hCG will also be provided in the methods of the present invention. It should be understood that a beneficial treatment may be achieved by administration of a PDE4 inhibitor alone.

To achieve the appropriate therapeutic outcome in the combination therapies contemplated herein, *i.e.*, to achieve an increase in the number of ovulatable oocytes in the mammal that is being treated, one would generally administer to the subject the FSH and the PDE-4 inhibitor composition. These compositions would be provided in a combined amount effective to produce the desired therapeutic outcome.

Typically, as indicated above, the FSH treatment is a course of daily administrations lasting between 7 to 12 days. The administration of the PDE-4 inhibitor may be administered concurrently with the FSH therapy. Alternatively, the PDE-4 inhibitor compositions may be taken prior to, or after, the FSH therapy. Furthermore, while the FSH therapy should preferably only be administered for any given period of 7 to 12 days during any given menstrual cycle, it is contemplated that the PDE-4 inhibitor may be administered continuously throughout the cycle as long as said administration does not cause deleterious side effects. Alternatively, the PDE-4 inhibitor may be administered less frequently than the FSH therapy.

In the other embodiments in which a third therapeutic agent is administered, the third agent may again be administered concurrently with one, other or both of the FSH and PDE-4 inhibitor therapeutic compositions or it may be administered prior to or after the FSH/PDE-4 therapies.

In embodiments all embodiments where two or more of the therapeutic compositions are administered separately, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the PDE-4-based agent and FSH administration and/or the third agent would still be able to exert an advantageously combined effect on the cell. In such instances, it is contemplated that one would administer all three compositions within about 12-24 hours of each other and, more preferably, within about 6-12 hours of each other, with a delay time of only about 12 hours being most preferred. In some situations, it may be desirable to extend the time period for treatment significantly, however, where several days (2, 3, 4, 5, 6 or 7) lapse between the respective administrations. Likewise, where the entire protocol is repeated, it may be desirable to either repeat the protocol through consecutive cycles or alternatively, the physician may determine that it is desirable to allow 1, 2 3, 4 or more cycles lapse between two treatment protocols of the present invention.

D. Patient Selection and Monitoring

The patients that receive the treatments of the invention are female patients between the age range of 20 to 45 year. Patient selection for the methods of the present invention may employ the same parameters as described in the PDR™ entries for use of FSH based therapies described above. For example, prior to

treatment the patient is subjected to a thorough gynecologic examination and endocrinologic evaluation, including an assessment of pelvic anatomy. Primary ovarian failure should be excluded by determining the basal serum gonadotropin levels and it should be ensured that the patient is not pregnant.

Throughout the treatment regimens of the present invention, the patient should be assessed prior to, during, and after, the therapy to monitor for the signs of OHSS. The symptoms of OHSS include but are not limited to abdominal pain, abdominal distention, gastrointestinal symptoms including nausea, diarrhea, severe ovarian enlargement, weight gain, dyspnea amd oliguria. Clinically, the symptom manifests in hypovolemia, hemoconcentration, electrolytic imbalance, ascites, hemoperitoneum, pleural effusions, hydrothorax acute pulmonary distress and thromboembolism. In the event that symptoms of OHSS occur during the administration of the FSH-based therapy or any other agent being administered for stimulation of follicular maturation, the administration should cease and the subject should be placed under medical supervision to determine whether hospitalization or other intervention is necessary. Other symptoms that may be used to monitor the FSH-based therapy include changes in vaginal cytology, appearance and volume of vaginal mucous, Spinnbarkeit and ferning of cervical mucus. These latter symptoms are indicative of the estrogenic effect of the therapy, and should be monitored because administration FSH will stimulate estrogen production. Preferably these estrogenic effects should be monitored in conjunction with more direct determinations of follicle development such as, e.g., determination of serum estradiol and ultrasonigraphy.

The clinical manifestations of ovulation, other than pregnancy, may be obtained either through a direct or an indirect measure of progesterone production. Such indicia include: a rise in basal body temperature, increase in serum progesterone, menstruation following a shift in body temperature. In conjunction with the above indicators of progesterone production, sonographic visualization of the ovaries may be used to assist in determining if ovulation has occurred. Such monographic monitoring may include evaluating fluid in the cul-de sac, ovarian stigmata and the presence of collapsed follicles. Sonographic determinations also will assist in determining whether the ovaries are enlarged in OHSS.

E. Use of the Oocytes for *In Vitro* Fertilization

The methods of the present invention are used to produce hCG ovulatable oocytes. The combined PDE-4 inhibitor/FSH hormonal treatment can be a short or long treatment, with or without pituitary down regulation, or with and without the use of GnRH antagonist; and with or without the use of hCG. The combined PDE-4 inhibitor/FSH therapy is administered until the follicles have matured sufficiently to be medium to full size follicles (size 10 to 25 mm, preferential 16 to 20 mm follicles). The oocytes from such follicles may be allowed to ovulate *in vivo*, either through the hCG surge from the patient's own menstrual cycle if the endogenous gonadotropin hormone production has not be suppressed, or by supplying to the patient an exogenous injection of hCG (e.g., 5000 to 10000 Units).

Alternatively, medium to full size follicles (size 10 to 25 mm, preferential 16 to 20 mm follicles) are harvested by aspiration under ultrasound guidance in order to be fertilized *in vitro*. The aspirated fluid is searched for cumulus oocytes complexes (COC) and once identified under the stereomicroscope (with or without the use of embryo filters), the COC is placed in culture. A wide variety of oocyte culture media or media components known to those of skill in the art. Such media may but does not necessarily have to contain human serum albumin (HSA).

Following or during *in vitro* culture, the oocytes may be fertilized by conventional IVF or by intracytoplasmatic sperm injection (ICSI) or by any other conventional fertilization methods leading to fertilized zygotes. The developing embryo may be transferred on day 1 to day 6 after fertilization, preferentially on day 2 to 3, either as single egg transfer or multiple egg transfer.

The patient can receive progesterone and/or oestrogen therapy before and after the egg transfer in individually designed protocols to prime and sustain appropriate receptive endometrial lineage.

The methods of the present invention produce an increased number of ovulatable oocytes for use in IVF procedures such as those described above. However, it may be that the methods of the present invention will also be useful in *in vitro* maturation of immature oocytes. In such embodiments, the oocytes are retrieved from antral follicles of the ovaries before being exposed to the mid-cycle surge of gonadotropins and are therefore characterized as immature or not fully matured

oocytes. Human oocytes as well as oocytes from other species will be recognized as having little or no cumulus expansion, a germinal vesicle and no polar bodies, and will readily be recognized as such by persons skilled in IVF-treatments. One potential use of the therapies of the present invention would be to replace CC or FSH in non-IVF protocols where such treatment may increase the maturation of a single or dominant follicle for natural fertilization rather than for harvesting for assisted reproductive technologies.

While much of the discussion herein has described the maturation of human follicles, it is contemplated that the methods of the present invention may be employed for producing oocytes for IVF of other mammals, such as a pet, e.g. a cat, a dog, or a guinea pig; or a zoo animal e.g. a primate. In further preferred embodiments, the mammal is part of the industry, preferably a farm animal such as cattle, a horse, a pig, a mink, a goat, or a sheep. It is in the most preferred embodiments, that the mammal is a human being. For *in vitro* maturation, the immature oocytes are treated *in vitro* with a PDE inhibitor or a PDE inhibitor and a gonadotropin hormone, in order to produce mature oocytes that can be fertilized.

F. Pharmaceutical compositions and kits

Pharmaceutical compositions for administration according to the present invention can comprise at least one gonadotropin hormone, preferably FSH according to the present invention in a pharmaceutically acceptable form optionally combined with a pharmaceutically acceptable carrier. These compositions can be administered by any means that achieve their intended purposes. Individualized amounts and regimens for the administration of FSH compositions for the stimulation of follicle maturation using the methods of the present invention can be determined readily by those with ordinary skill in the art using the guidance provided by the Physician's Desk Reference for the use of such compositions in treating anovulatory disorders and for their use in assisted reproduction technologies. As discussed above, those of skill in the art could initially employ amounts and regimens of FSH currently being used in such medical contexts. To this effect, those skilled in the art are specifically referred to each of the entries in the Physician's Desk Reference discussed above and those entries are incorporated herein by reference in their entireties for teaching methods and compositions for the administration of agents such

as Fertinex™, Gonal F™, Bravelle™ and the like discussed herein above. Each of those entries in the Physician's Desk Reference provide exemplary guidance as to types of formulations, routes of administration and treatment regimens that may be used in administering FSH. Any of the protocols, formulations, routes of administration and the like described therein can readily be modified for use in the present invention.

Compositions within the scope of this invention include all compositions comprising at least one PDE-4 inhibitor according to the present invention in an amount effective to achieve its intended purpose of stimulating, inducing or otherwise increasing the number of ovulatable oocytes in an animal, either when administered alone or more preferably, when administered in combination with a low dose of FSH. The PDE-4 inhibitors and/or the other active agents used in the methods of the present invention may be administered by any means normally employed for such administration. Those of skill are particularly referred to U.S. Patent Application No. 60/470,434 entitled Ihibitors of PDE enzymes in infertility,"; U.S. Patent Publication No. 20020103106 and PCT/EP01/14730, each of which describe amounts and routes of administration of PDE inhibitors in fertility related applications. Most preferably, the compositions used in the present invention are administered orally.

While individual needs vary, determination of optimal ranges of effective amounts of each component is within the skill of the art. Typical dosages of the FSH comprise about 5 IU FSH/day to about 75 IU FSH/day, for a 7 to 12 day period. Typical doses of he PDE-4 inhibitor may vary from about 5 mg/day to about 100 mg/day or more. While continuous, daily administration of the PDE-4 is contemplated, it may be desirable to cease the PDE-4 administration at the same time as the FSH administration is ceased. Of course, while FSH therapy is traditionally given for a period of 7 to 12 days, it may be that in the context of the present invention only a single or a few low doses of FSH are needed to effect the therapeutically beneficial outcome of increased follicle maturation. Therapy should be halted in the event that symptoms of OHSS are observed.

It is understood that the suitable dose of a composition according to the present invention will depend upon the age, health and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect

desired. However, the most preferred dosage can be tailored to the individual subject, as is understood and determinable by one of skill in the art, without undue experimentation. This typically involves adjustment of a standard dose, *e.g.*, reduction of the dose if the patient has a low body weight.

As discussed above, the total dose required for each treatment may be administered in multiple doses or in a single dose. The compositions may be administered alone or in conjunction with other therapeutics directed to the disease or directed to other symptoms thereof.

As is apparent from the disclosure presented herein, in a broad aspect the present application contemplates clinical application of a combination therapy comprising a first composition that contains a PDE inhibitor, and a second composition that contains FSH. Therefore, the compositions should be formulated into suitable pharmaceutical compositions, *i.e.*, in a form appropriate for *in vivo* applications in such combination therapies. Generally, this will entail preparing compositions that are essentially free of pyrogens, as well as other impurities that could be harmful to humans or animals. The FSH formulations may be formulated akin to the currently available FSH preparations discussed herein throughout. The PDE-4 inhibitor formulations may be formulated similarly to e.g., formulations of Viagra™, which is a well-known PDE-5 inhibitor.

One will generally desire to employ appropriate salts and buffers to render the compositions stable and allow for uptake of the compositions at the target site. Generally the hormone compositions of the invention are provided in lyophilized form to be reconstituted prior to administration and the PDE-4 inhibitor compositions are likely formulated into tablet form. Buffers and solutions for the reconstitution of the hormones may be provided along with the pharmaceutical formulation to produce aqueous compositions of the present invention for administration. Such aqueous compositions will comprise an effective amount of each of the therapeutic agents being used, dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. Such compositions also are referred to as inocula. The phrase "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce adverse, allergic, or other untoward reactions when administered to an animal or a human. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and

antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the therapeutic compositions, its use in therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

The active compositions of the present invention include classic pharmaceutical preparations of FSH, which have been discussed herein as well as those known to those of skill in the art. PDE-4 inhibitors also are known to those of skill in the art. Administration of these compositions according to the present invention will be via any common route so long as the target tissue is available via that route. Most commonly, these compositions are formulated for oral administration. However, other conventional routes of administration, *e.g.*, by subcutaneous, intravenous, intradermal, intramusclar, intramammary, intraperitoneal, intrathecal, intraocular, retrobulbar, intrapulmonary (*e.g.*, term release), aerosol, sublingual, nasal, anal, vaginal, or transdermal delivery, or by surgical implantation at a particular site also may be used particularly when oral administration is problematic. The treatment may consist of a single dose or a plurality of doses over a period of time.

The active compounds may be prepared for administration as solutions of free base or pharmacologically acceptable salts in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions also can be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained,

for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

The compositions of the present invention may be formulated in a neutral or salt form. Pharmaceutically-acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups also can be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug release capsules and the like. For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration.

"Unit dose" is defined as a discrete amount of a therapeutic composition dispersed in a suitable carrier. Examples of preferred doses of the FSH and the PDE inhibitors have been discussed above. Parenteral administration of one or both of the therapeutic compounds may be carried out with an initial bolus followed by continuous infusion to maintain therapeutic circulating levels of drug product. Those of ordinary skill in the art will readily optimize effective dosages and administration regimens as determined by good medical practice and the clinical condition of the individual patient.

The frequency of dosing will depend on the pharmacokinetic parameters of the agents and the routes of administration. The optimal pharmaceutical formulation will be determined by one of skill in the art depending on the route of administration and the desired dosage. See for example Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publ. Co, Easton PA 18042), incorporated herein by reference. Such formulations may influence the physical state, stability, rate of *in vivo* release and rate of *in vivo* clearance of the administered agents. Depending on the route of administration, a suitable dose may be calculated according to body weight, body surface areas or organ size. Further refinement of the calculations necessary to determine the appropriate treatment dose is routinely made by those of ordinary skill in the art without undue experimentation, especially in light of the dosage information and assays disclosed herein as well as the pharmacokinetic data observed in animals or human clinical trials.

Appropriate dosages may be ascertained through the use of established assays for determining blood levels in conjunction with relevant dose response data. The final dosage regimen will be determined by the attending physician, considering factors which modify the action of drugs, *e.g.*, the drug's specific activity, severity of

the damage and the responsiveness of the patient, the age, condition, body weight, sex and diet of the patient, the severity of any infection, time of administration and other clinical factors. As studies are conducted, further information will emerge regarding appropriate dosage levels and duration of treatment for specific diseases and conditions.

It will be appreciated that the pharmaceutical compositions and treatment methods of the invention may be useful in fields of human medicine and veterinary medicine. Thus the subject to be treated may be a mammal, preferably human or other animal. For veterinary purposes, subjects include for example, farm animals including cows, sheep, pigs, horses and goats, companion animals such as dogs and cats, exotic and/or zoo animals, laboratory animals including mice rats, rabbits, guinea pigs and hamsters; and poultry such as chickens, turkey ducks and geese.

The present invention also contemplated kits for use in the treatment of fertility disorders. Such kits include at least a first composition comprising an FSH in a pharmaceutically acceptable carrier, and a second composition comprising at least one PDE-4 inhibitor in a pharmaceutically acceptable carrier. The kits may additionally comprise solutions or buffers for effecting the delivery of the first and second compositions. The kits may further comprise additional compositions which contain further PDE inhibitors e.g., additional PDE-4 inhibitors or additional PDE 1 or PDE-5 inhibitors and/or further hormones such as e.g., hCG, LH and the like. The kits may further comprise catheters, syringes or other delivering devices for the delivery of one or more of the compositions used in the methods of the invention. The kits may further comprise instructions containing administration protocols for the therapeutic regimens.

G. Examples

The following example(s) is included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the example(s) that follows represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes

can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Materials & Methods

Animals: Immature Sprague Dawley CD (SD) BR female rats, weighing 36-39 g on receipt were used. The animals were housed in a room under the following constant environmental conditions: temperature 22°C ± 2, relative humidity 55% ± 10, 15-20 air changes per hour (filtered on HEPA 99.99%) and artificial light with a 12-hour circadian cycle (7h00 – 19h00).

For the entire duration of the study the rats were kept in wire cages (cm. 40.5x38.5x18h) with stainless steel feeders and fed on a standard pelleted diet and water "ad libitum".

Chemicals: Human recombinant follicle stimulating hormone (r-hFSH) and human recombinant chorionic gonadotrophin (r-hCG) were supplied by Laboratoires Serono Aubonne (LSA, Aubonne, Switzerland). Test compounds were either synthesized based on published compound synthetic methods or purchased from commercial sources. In particular, those of skill are referred to WO9501338 which teaches methods of making Roflumilast, J.Med. Chem. 1994, 37,1696-1703 for a detailed description of methods of making Piclamilast, J.Med. Chem. 1998, 41,821-835 for a description of methods for making Ariflo/Cilomilast, WO97/15561 for methods of making Mesopram, and EP0470 805 B1 for methods of making Filaminast. Such methods may be modified for producing other PDE inhibitors. In addition, U.S. Patent Application No. 60/470,434 entitled Inhibitors of PDE enzymes in infertility," U.S. Patent Publication No. 20020103106, and PCT/EP01/14730 are incorporated herein by reference as teaching other related such compounds that may be used herein.

In vivo Rat Follicle Maturation Assay: Immature female rats arrived on Friday of the week prior to the experimentation at 18-19 days of age along with a lactating female (ten pups per lactating female). All rats were weaned from the mother on the following Monday (21-22 days old) and were randomly sorted into the experimental groups (6-8 animals/group).

The rats were subcutaneously injected (in the scruff of the neck) twice a day for two days (first injection: 8.30 – 9.00 and second injection 15.30 – 16.00) with r-hFSH or vehicle (PBS) at a volume of 250 µL/injection. The doses of FSH injected were either suboptimal (606 ng/rat total dose split over four injections; indicated as 'Low FSH' in Figures) or high (2424.8 ng/rat total dose split over 4 injections; indicated as 'High FSH' in Figures) as a positive control.

In addition to the above injections, rats were also injected subcutaneously with the test PDE-4 inhibitor compounds or vehicle twice a day for 2 days at indicated doses (mg/kg/injection), concomitantly with the FSH injections. These compounds were diluted in either NP3S (n-methyl-2-pyrrolidone 5%, polyethylene glycol 400 30%, polyethylene glycol 200 25%, propylene glycol 20%, saline 20%) or aqueous vehicles. Therefore, the total number of injections received by each rat to promote follicle growth was eight, including four injections of r-hFSH or r-hFSH vehicle plus four injections of test compound or test compound vehicle.

On day 2 of the experiment and along with the final r-hFSH injection, the rats were also treated with a single subcutaneous injection of r-hCG (1430 ng/rat) to induce ovulation of all or most of the matured follicles.

At 10h00 of the morning following r-hCG administration, rats were euthanized by CO2 asphyxia. The animals were laid on their backs and undersides were sprayed with ethanol to both sterilize and keep the hair from falling out in the dissection of the animals. With the aid of scissors and forceps the skin and muscle were cut starting from the pubic symphisis with aboral-oral direction up to the sternum. The internal organs were exposed and the intestine was moved to one side. The ovaries, the uterine horns and the uterus body were removed clipping away the fat and the connective tissue. The entire reproductive tract was then placed into a well in a 24 well plate containing PBS (1 animal/well).

After all the animals were sacrificed and the ovaries were harvested, the oviducts were gently removed from the ovaries, dipped in PBS and placed on a microscope slide. The ovary was then taken out, cleaned and placed into PBS for weighing (the uterus was discarded).

Pairs of oviducts were placed on one slide and then a slide was placed on top of the first slide using a piece of tape to secure the frosted ends of the slides

together. After the oviducts were placed on the bottom slide, the top slide was folded over and the non-frosted end was then taped, compressing the oviducts between the two slides. The oviducts were then examined by a light microscope under contrast phase conditions (at a minimum of 40x magnification) and the ova, if any, present in the two ampullae for each rat were counted. The results were graphed as the average number of oocytes ovulated per rat. Error bars reflect the standard error of the mean for each group.

In order to measure cAMP in cell lines, JC-410 porcine granulosa, cells were used. These cells were obtained from Dr. Jorge Chedrese (University of Saskatchewan). The cells were maintained in DMEM/F12 supplemented with 5% newborn calf serum (Gibco) and 5 μg/ml of insulin (Gibco). Stable cell lines were established by transfecting the cDNAs for the human LH and FSH receptors into the cells using standard transfection techniques and selection with 300 μg/ml of Geneticin (Gibco). Following selection, the cells were maintained in the same concentration of Geneticin. For cAMP determinations, the cells were plated at a density of 25,000 cells per well, in 96 well plates, one day prior to the assay. The following day, the cells were stimulated for 1 hour with increasing doses of the inhibitor molecules in the presence, or absence, of 1 nM FSH, as indicated. All compounds were diluted in assay buffer (DMEM/F12, 0.1% BSA (Sigma), 100 μM IBMX) containing 4% DMSO (0.5% final concentration in the assay). After a 1 hour stimulation, the cells were lysed and cAMP was assayed using the Tropix cAMP-Screen assay (Applied Biosystems), according to the manufacturers protocol.

Measurements of cAMP in primary ovarian dispersate cultures were monitored as follows. Ovaries were harvested from Sragué-Dawley rats (22 days old) and decapsulated under a dissecting microscope in 3-5 ml of digestion media. The digestion media consisted of assay media (McCoy's 5A media supplemented with 1 mg/ml BSA (Sigma), 5 μg/ml of gentamycin and 3 μg/ml of amphotericin B (Gibco)), containing 8 mg/ml of collagenase (Sigma) and 0.05% DNase (Invitrogen). After decapsulating, the ovaries were dissected into small pieces using 27 gauge needles, transferred to a 15 ml tube and digested for 45 min at 37°C, while shaking. The digested tissue was filtered through a 70 μm Nalgene Filter and the filtrate was centrifuged at 1000 rpm for 5 min. The pellet was washed with assay media and centrifuged a second time. The resulting pellet was suspended in 2 ml of growth

media (McCoy's 5A media supplemented with 5% FBS, 5 μg/ml gentamycin, 3 μg/ml amphotericin B (Gibco)) and the cells were counted. The cells were diluted in growth media, plated at 25-30,000 cells/well and cultured for 48 hours prior to stimulation. The cells were stimulated and cAMP was assayed as described above for the cell line measurements.

Results

A panel of PDE-4 inhibitors were tested for their ability to induce cAMP in FSH treated, or untreated, rat granulosa cells and/or human FSH receptor-expressing porcine granulosa cell line JC410 (JC410FSHR) *in vitro*. Of the PDE-4 inhibitors, some were highly active in inducing cAMP, some were of intermediate activity and some had little or no activity in this model. Notably, in the absence of FSH, the PDE-4 inhibitors had little or no capacity to induce cAMP (see FIG. 1A-C). The potency of the inhibitors was the same regardless of the source of the granulosa cells, *i.e.*, it was relatively similar in both the cell line and the primary granulosa cells.

In vivo, two exemplary PDE-4 inhibitors, Piclamilast and Roflumilast, increased FSH-induced follicle maturation. Low concentrations of Piclamilast (0.08 and 0.4 mg/kg) were was not sufficient to induce follicle maturation in the absence of a low level of exogenous FSH (FIG. 4), however, at the higher concentration of 2 mg/kg Piclamilast did induce follicle maturation (see FIG. 4). When Piclamilast is administered in the presence of low doses of FSH there was a very marked induction in follicle maturation as evidenced by the increase in number of ovulatable oocytes (FIG. 5).

The above results show that PDE-4 inhibitors can, when administered to mammals along with a suboptimal doses of FSH, lead to an increase in the number of hCG-ovulatable oocytes. Methods and compositions for exploiting this finding have been described herein above.

All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without departing from the

concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

The references, patents and patent publications cited herein throughout, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are all specifically incorporated herein by reference.

CLAIMS

What is Claimed Is:

1. A method of increasing follicle maturation comprising treating a mammal with a phosphodiesterase (PDE) inhibitor in an amount effective to stimulate follicular growth and maturation.

2. A method of *in vitro* oocyte maturation comprising treating an oocyte *in vitro* with a PDE inhibitor in an amount effective to cause oocyte maturation.

ABSTRACT

The present invention is directed to methods of increasing oocyte production in a mammal. More specifically, the specification describes methods and compositions for inducing follicular maturation using a PDE inhibitor. The inhibitor may be used alone at high doses. Alternatively, the follicular maturation is achieved by combining a low dose of FSH with the PDE-4 inhibitor treatment.

FIG. 1A-FIG. 1C

FIG. 1A

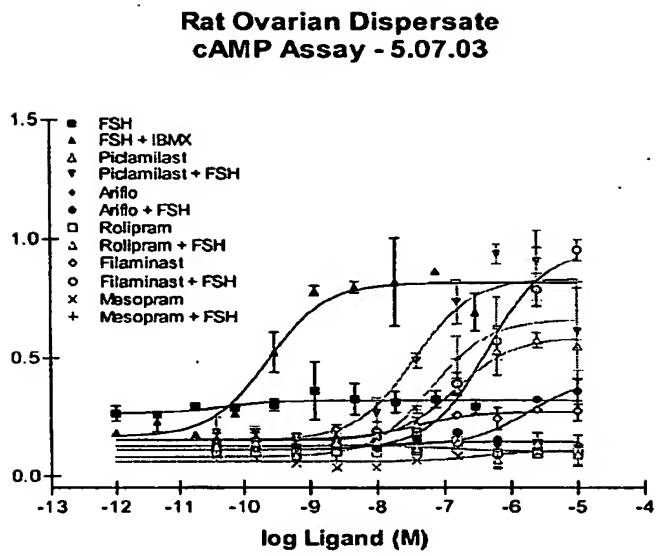
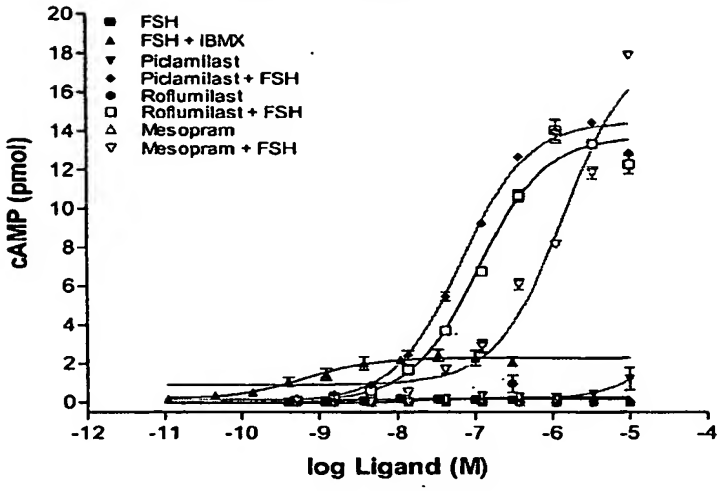
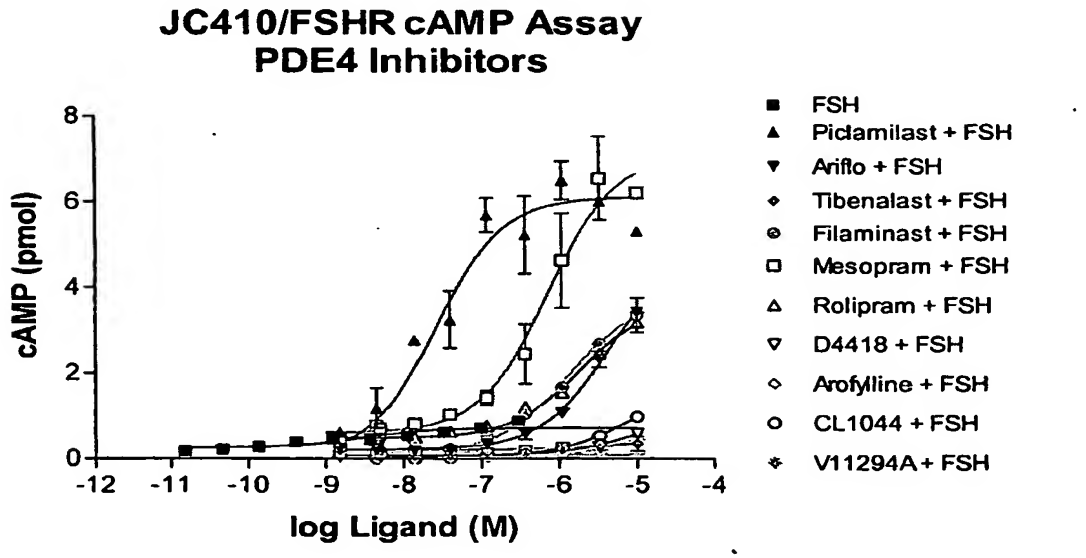


FIG. 1B





	FSH	Pidamilast + FSH	Ariflo + FSH	Tibenalast + FSH	Filaminast + FSH	Mesopram + FSH	Rolipram + FSH	D4418 + FSH	Arofylline + FSH	CL1044 + FSH	V11294A + FSH
Bottom	0.2267	0.04304	0.2039	0.2114	0.1291	0.0952	0.1765	0.05229	0.01747	0.0469	0.1732
Top	0.7132	0.112	0.632	0.4134	3.733	7.139	3.837	1.088	0.1115	1.888	0.08579
LogEC50	-6.919	-7.573	-6.398	-6.179	-6.877	-6.139	-6.732	-6.974	-6.089	-6.123	-6.387
EC50	1.202e-007	2.670e-008	4.030e-008	6.661e-008	1.329e-006	7.276e-007	1.850e-007	1.062e-007	8.129e-008	7.523e-008	4.091e-007

FIG. 1C

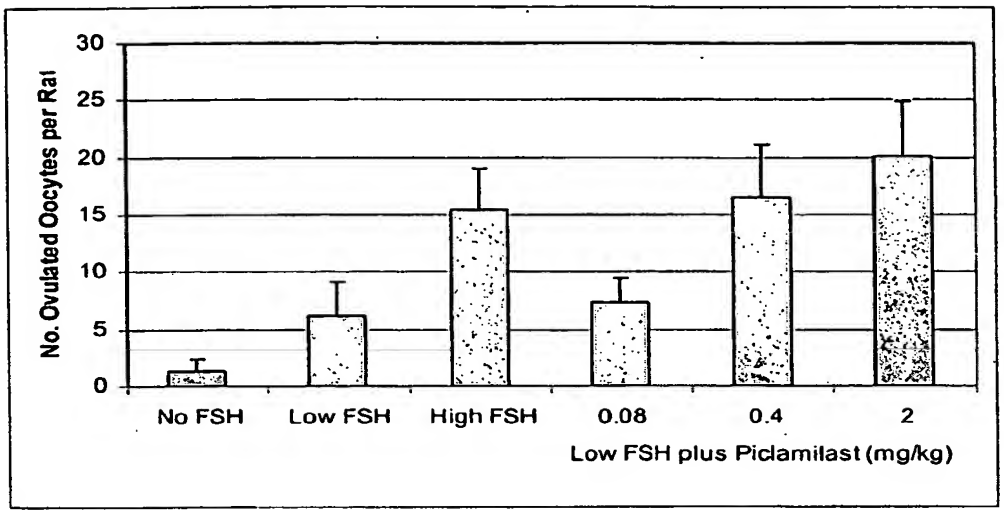

No. Ovulated Oocytes per Rat
30
25
20
15
10
5
0
No FSH
Low FSH
High FSH
0.08
0.4
2
Low FSH plus Piclamilast (mg/kg)

FIG. 2

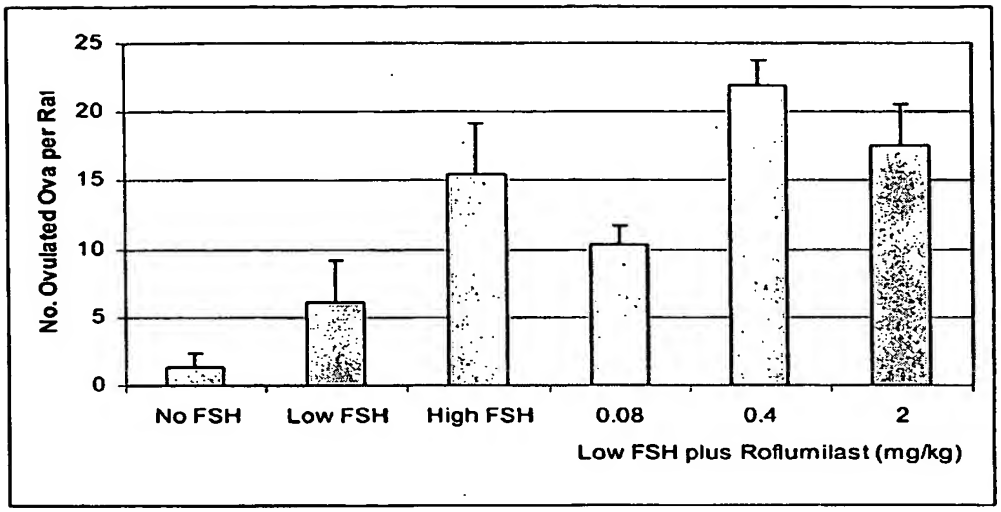
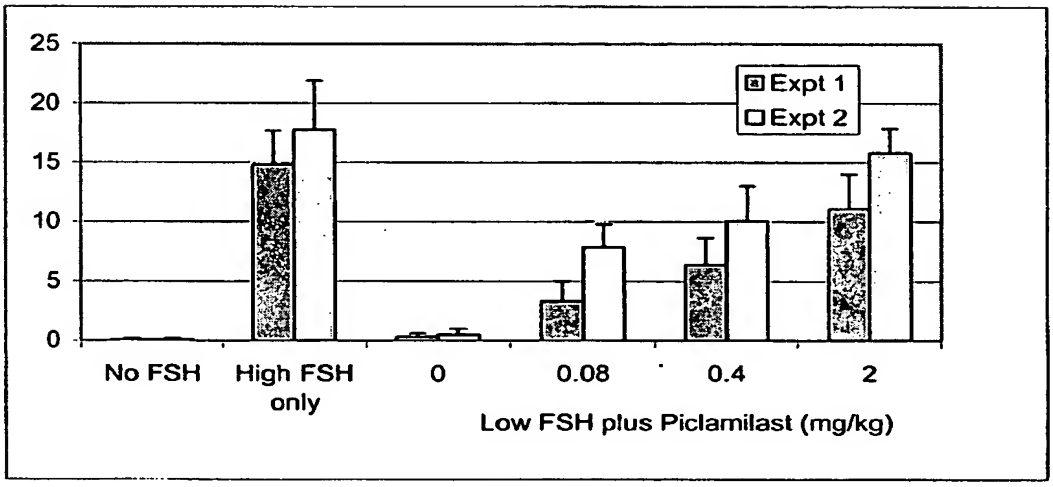

25
20
15
10
5
0
No. Ovulated Ova per Rat
No FSH
Low FSH
High FSH
0.08
0.4
2
Low FSH plus Roflumilast (mg/kg)

FIG. 3

FIG. 4


25
20
15
10
5
0
Expt 1
Expt 2
No FSH
High FSH only
0
0.08
0.4
2
Low FSH plus Piclamilast (mg/kg)

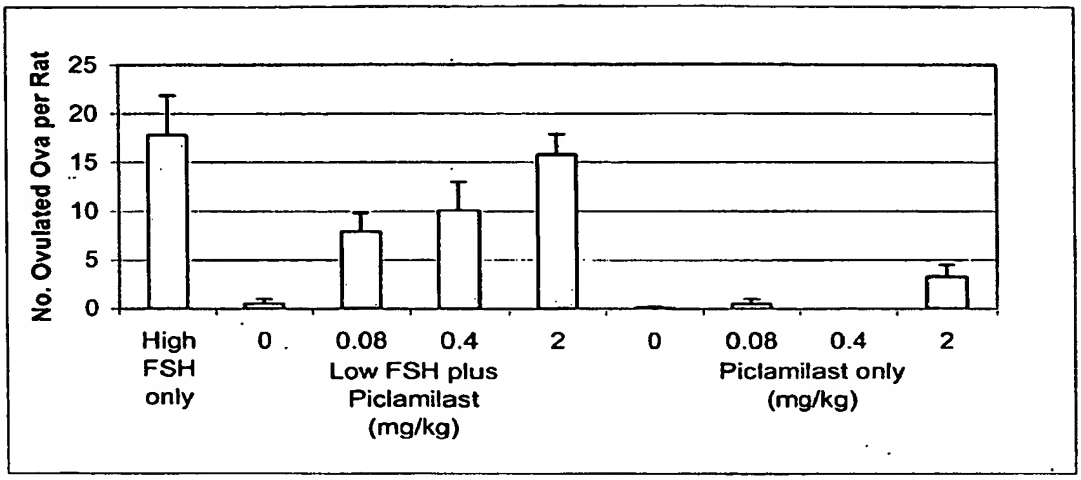

No. Ovulated Ova per Rat
25
20
15
10
5
0
High FSH only
0
0.08
0.4
2
Low FSH plus Piclamilast (mg/kg)
0
0.08
0.4
2
Piclamilast only (mg/kg)

FIG. 5

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